Serum C-reactive protein and progesterone profile in peripartum bitches and evaluation of CRP as a marker of impending parturition

Running head: CRP and progesterone around bitches parturition

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Abstract

C-reactive protein (CRP) is one of the major acute phase proteins in dogs; it is produced by the liver and rapidly increases in response to an inflammatory stimulus. The aim of this study was to measure CRP concentrations around parturition and to verify whether this protein could be useful, together with progesterone (P), to detect the end of pregnancy in bitches. CRP and P concentrations were measured on 66 serum samples from 28 healthy pregnant bitches, collected between -5 and +2 days from parturition. The effect of 'days from parturition', parity, and litter size on P and CRP concentration was analyzed. P and CRP values were significantly affected by 'days from parturition'. While P showed the expected decline during the last days of pregnancy, CRP concentration was above the normal range from the day of parturition onwards, beginning to increase at day -1. The CRP concentration profiles during the days around parturition have not been previously reported in the dog. However, the late rise and the low magnitude of increase make CRP difficult to use in clinical practice to assess the end of pregnancy in the bitch.

Keywords

Dog; Parturition; C-reactive protein; Progesterone

1. Introduction

C-reactive protein (CRP) is an acute-phase protein (APP) that is mainly produced in the liver upon stimulation by proinflammatory cytokines. The acute phase response is a nonspecific reaction that is triggered by any tissue injury and develops following either infectious, immunologic, neoplastic, traumatic or other causes (Ceron et al., 2005). APPs are mediators and inhibitors of inflammation, and express their protective effect through the opsonization of apoptotic or necrotic cells, by binding to bacterial proteins and by influencing the immune response which accompanies inflammation (Gabay and Kushner, 1999). CRP shows an early and strong response in dogs: a 20- to 100-fold increase, depending on the cause of inflammation, can be detected after four hours, with a peak concentration at 24-48 hours (Ceron et al., 2005). CRP can be evaluated in clinical exams as a marker of both acute and chronic inflammatory disorders (Ceron et al., 2008). A rise in CRP concentration has been detected in the first (Eckersall et al., 1993) or second (Kuribayashi et al., 2003) third of pregnancy in the bitch, followed by a decline before parturition. A second increase after parturition has been occasionally observed (Eckersall et al., 1993). Implantation of the developing embryo in the endometrium and placental development were suggested as the likely cause of an acute phase response (Eckersall et al., 1993) and it has also been ascribed to 'the influence of endocrine hormones during pregnancy' (Kuribayashi et al., 2003). In the past, the analysis of CRP and other APPs concentration was proposed as a method for early pregnancy diagnosis in the bitch (Evans and Anderton, 1992; Vannucchi et al., 2002).

Although the exact mechanisms responsible for parturition are still to be elucidated, the presence of an inflammatory response in the myometrium has been ascertained in women (Thomson et al., 1999; Mendelson and Condon, 2005; Leong et al., 2008).

A reliable estimate of parturition date is rather difficult in the bitch when only mating dates are available to the clinician. A precise prediction of impending parturition could be very useful in order to avoid long observation periods and it is critical when planning a cesarean section. Serum progesterone concentration declines towards the end of pregnancy (Onclin and Verstegen, 1997)

and some cut-off values have been calculated; when P is lower than the cut-of value, parturition is likely to occur within a given time interval (Rota et al., 2015; De Cramer and Nöthling, 2018). However, large individual variability exists in progesterone concentration, particularly in the last days of pregnancy (Rota et al., 2015; De Cramer and Nöthling, 2018), and other easily measurable parameters that could mark the end of pregnancy would be very useful in clinical practice.

Since CRP measurement is part of the routine biochemical profiles performed by many veterinary laboratories, the aim of this study was to measure peripartum CRP concentration in the bitch and to assess its use in clinical practice to detect the end of pregnancy in the bitch.

2. Materials and Methods

Animals and samples

Twenty-eight healthy pregnant bitches that whelped live puppies were included in the study. The bitches, of various breed [Staffordshire Bull Terrier (N=6), Flat Coated Retriever (4), Boxer (4), Jack Russell Terrier (3), Bouvier des Flandres (2), Australian Shepherd (2), and one each of the following: American Staffordshire Terrier, Bloodhound, Bassett Hound, Labrador Retriever, Golden Retriever, Samoyed, Rough Collie] and parity, ranging in age from 2 to 8 years (3.9±1.6 mean ± SD), had been presented to the veterinary hospitals of the University of Padova or Torino for pregnancy monitoring and parturition assistance, in the period from June 2017 to October 2017. Blood had been collected by cephalic venipuncture for routine progesterone assay and for routine biochemistry evaluation and sera remnants had been stored frozen at -20°C.

The case sheet of each bitch reported the day of parturition and the number of delivered puppies. Ex post selection of sera from samples collected between five days before and two days after parturition was carried out. Written informed consent to use the stored samples was obtained by dog owners.

The study was performed in accordance with the guidelines for the care and use of animals of the Department of Veterinary Science of the University of Turin and of the Department of Animal Medicine Production and Health of Padova.

89 *Measurement of Progesterone and CRP*

90 CRP was measured with a turbidimetric method (BT1500®, Biotecnica instruments SpA,
91 Roma, Italy); normal reference values are considered in the interval 0- 1.07 mg/dl. The assay had
92 been previously correlated with a canine CRP turbidimetric assay (Randox canine CRP reagents,
93 RANDOX, Milan, Italy) validated for the canine species (Kjelgaard-Hansen et al., 2003).
94 Repeatability was CV<7.5%; Linearity (O/A) y=0.9795x – 0.0074 R^2=0.9935. The relationships
95 between the two kits was obtained by the analysis of 91 samples and described by the linear
96 regression as follows: y=1,5135x – 0,0123. R^2= 0.9431.
97 Progesterone was measured by Chemiluminescence immunoassay (CLIA) (Immulite 2000®;
98 Siemens Diagnostics, Flanders, NJ, USA) (Kutzler at al., 2003).
99 *Statistical analysis*

100 Statistical analysis was performed with a repeated mixed linear model where days from
101 parturition (days -5 to +2), parity (primiparous vs multiparous), and number of delivered puppies
102 (<4 vs 4-8 vs >8) were considered as fixed effects and dog was considered as random and repeated
103 effect. Hypotheses of linear model on residuals were graphically assessed. Post hoc pairwise
104 contrasts among levels were calculated using Bonferroni correction. Data were reported as least-
105 squares means ± standard error (ls-means±SE). Day 0 was the day of parturition. Significance was
106 set at $P<0.05$. Data are presented as least-squares mean ± standard error.
107 Spearman rank correlation was calculated between CRP and Progesterone values.
108 Using the threshold of 2 ng/ml to identify bitches at term (Concannon et al., 1977), the animals
109 were divided into two groups (at term/not at term). The ability of CRP to distinguish between
110 bitches at term and not at term was evaluated by receiver operating characteristic (ROC) curve
111 analysis. Sensitivity, specificity and cut-off value of this potential marker were calculated (with
112 95% confidence interval, CI). To select the optimal cut-off value, with 95% CI, Youden's Index
113 was calculated. The value of the area under the curve (AUC) as a criterion of the accuracy of the

marker was defined as low (0.5-0.7), moderate (0.7-0.9) and high (>0.9) (Kjelgaard-Hansen et al., 2003).

All analyses were performed with statistical software packages SAS V.9.3 (SAS Institute Inc., Cary, NC) and MedCalc v.12.4.0 (Ostend, Belgium).

3. Results

P and CRP concentration were measured on a total number of sixty-six serum samples. The number of samples for each of the eight days of observation was distributed as shown in Fig. 1.

CRP concentrations ranged from 0/0.04 mg/dl to 2.74 mg/dl and were significantly affected by 'days from parturition' (P=0.0195), parity (P=0.044) and number of delivered puppies (P=0.0036). Serum values showed a significant increase over time (Table 1), however pairwise contrasts did not reveal significant daily differences. Mean CRP concentrations were above the normal range from day 0 onwards, beginning to increase at day -1.

Primiparous bitches had significantly higher CRP concentrations than pluriparous ones (Fig. 2). Bitches that whelped less than 4 puppies had significantly lower CRP concentrations than bitches with larger litters (Fig. 3).

Progesterone concentration was significantly affected by 'days from parturition' (P<0.0001) and showed the expected decline during the last days of pregnancy. The value at day -1 (2.74±0.49 ng/ml did not differ significantly from the values at days -3, -2 and 0 (4.44±0.47, 3.90±0.50 and 0.60±0.49 respectively) (Fig. 4). Postpartum values were below 1 ng/ml and did not differ between days. Neither parity nor litter size significantly affected progesterone concentration.

An inverse correlation was found between CRP and progesterone (r= -0.52; P<0.001, Fig. 5).

ROC curve results are reported in Fig. 6. The area under the curve (AUC) was 0.73, with 95% CI (0.591-0.835; P=0.001), meaning that CRP has a moderate accuracy as a marker of impending parturition. The cutoff value that maximizes Youden's Index is 1.41 mg/dl, with a sensitivity of 87.5% (71-96.5; 95% CI) and a specificity of 56% (34.9-75-6; 95% CI).

4. Discussion

Serum progesterone concentrations towards the end of pregnancy can help clinicians to assess impending canine parturition (Rota et al., 2015; De Cramer and Nöthling, 2018), but our data show that the progesterone concentrations are not significantly different in the last three days of pregnancy. Other easy-to measure serum parameters could be useful to increase the accuracy of the diagnosis and CRP, that can be assayed in any facility having instruments for biochemistry serum analysis, could have represented an option. Our hypothesis was that uterine inflammation is a characteristic feature of labour and parturition in bitches, as in women (Thomson et al., 1999; Mendelson and Condon, 2005; Leong et al., 2008), and that CRP, which is a strong and early marker of inflammation in dogs (Ceron et al., 2005; Ceron et al., 2008) could also be a marker of the end of pregnancy. Serum concentrations of CRP during canine pregnancy have been reported in various studies, with contrasting results, but daily values around parturition have not been previously measured in the dog. Different methods for CRP determination were used, resulting in different values. Our data are similar to those of Eckersall et al. (1993) who like us used the technique of immunoturbidimetry, although with a different commercial kit. With a 'solid sandwich' immunoassay, Ulutas et al. (2009) obtained different absolute values of CRP concentration. Although technical differences may explain these differences, they hardly explain the different serum profiles found in previous investigations. Some studies revealed a strong, 7-10 fold, increase of CRP serum concentrations beginning around the third-fourth week after ovulation, followed by a decline in the last third of pregnancy (Eckersall et al., 1993; Kuribayashi et al., 2003). In six out of nine beagle bitches, an increase after parturition was also detected (Eckersall et al., 1993). Other investigations (Concannon et al., 1996; Ulutas et al., 2009) did not detect such a serum profile. Ulutas et al. (2009) observed a significantly higher CRP serum concentration in pregnant bitches than in bitches in proestrus, but the increase in CRP concentrations was very low, both in the first and in the second half of pregnancy.

164 The rate of the increase in serum CRP concentration that we detected from the day of parturition
165 onward is lower than the value reported by Eckersall et al. (1993), who observed a three- to ten-fold
166 rise.
167 Our data did not reveal any sharp increase of CRP serum values, making it difficult to use this
168 parameter as a marker of impending parturition. However this result is consistent with the trend of
169 progesterone concentration and a weak but significant negative relationship between CRP and
170 progesterone was present, meaning that CRP concentration is going to increase at the decrease of
171 progesterone concentration.
172 The peripartum pattern of CRP concentration that we observed in the bitch is similar to what has
173 been reported for other species, irrespective of different placental types and if they are unitocous or
174 polytocous. An increase of CRP serum concentrations has indeed been detected in sows, where it is
175 evident from the day of parturition until day 7 after farrowing (Wierzchosławski et al., 2018). CRP
176 increased just before delivery (≤2days) in pregnant mares and decreased from 7 days after
177 parturition (Yamashita et al., 1991). In women, the CRP concentrations are generally higher during
178 pregnancy, and a significant increase can be observed in the postpartum period, approximately10-
179 folds as high as the concentrations during the second and third trimester values (Skarżyńska et al.,
180 2018). CRP concentration was also significantly higher during the first month after calving than in
181 the last trimester of pregnancy in cows (Dębski et al., 2016).
182 These and our data show that parturition causes an increase in CRP concentrations, likely due to
183 uterine physiological inflammatory conditions (Thomson et al., 1999; Leong et al., 2008). In
184 primiparous bitches the inflammatory response can be higher because involving tissues that for the
185 first time undergo modifications correlated to the end of pregnancy. Also the influence of litter size
186 on CRP level is consistent with a higher inflammatory response provoked by a higher number of
187 feto-placental units.
188 The ROC curve analysis showed that CRP is a marker of impending parturition that has a
189 'moderate' accuracy (Kjelgaard-Hansen et al., 2003). Despite a rather good sensitivity (87.5%), it

has a low specificity (56%), meaning that when CRP concentration drops below the cutoff value (identified as 1.41 mg/dl) a clinician can distinguish a true impending parturition in 87.5% of cases, while the bitch not at term will be correctly identified in 56% of cases.

The late rise and the low magnitude of serum concentrations of CRP found in this study lead to conclude that this parameter cannot be used alone in clinical practice to assess the end of pregnancy in the bitch. However, further studies are needed in order to correlate CRP concentration with other parameters of impending parturition, and especially with the physiological or pathological outcome of parturition.

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Conflict of Interest Statement

The authors have no conflict of interest to declare.

References

Ceron, J.J., Eckersall, P.D, Martýnez-Subiela, S., 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet. Clin. Pathol. 34, 85-99.

Ceron, J.J., Martinez-Subiela, S., Ohno, K., Caldin, M., 2008. A seven-point plan for acute phase protein interpreation in companion animals. Vet. J. 177, 6-7.

Concannon, P.W., Gimpel, T., Newton, L., Castracane, D., 1996. Postimplantation increase in plasma fibrinogen concentration with increase in relaxin concentration in pregnant dogs. Am. J. Vet. Res. 57, 1382-1385.

Concannon, P.W., Powers, M.E., Holder, W., Hansel, W., 1977. Pregnancy and parturition in the bitch. Biol. Reprod. 16,517-526.

215 Dębski, B., Nowicki, T., Zalewski, W., Ochota, M., Mrowiec, J., Twardoń, J., 2016. Evaluation of
216 acute phase proteins in clinically healthy dairy cows in perinatal period and during lactation. Pol.
217 J. Vet. Sci. 19, 519-523.
218 De Cramer, K.G.M., Nöthling, J.O., 2018. The precision of predicting the time of onset of
219 parturition in the bitch using the level of progesterone in plasma during the preparturient period.
220 Theriogenology. 107, 211-218.
221 Eckersall, P.D., Harvey, M.J.A., Fergunson, J.M., Renton, J.P., Nickson, D.A., Boyd, J.S., 1993.
222 Acute phase protein in canine pregnancy (*Canis familiaris*). J Reprod.Fertil.Suppl. 47, 159-164.
223 Evans, J.M., Anderton, D.J., 1992. Pregnancy diagnosis in the bitch: the development of a test
224 based on the measurement of acute phase proteins in the blood. Ann. Zootech. 41, 397-405.
225 https://hal.archives-ouvertes.fr/hal-00888856.
226 Gabay, C., Kushner, I., 1999. Acute-phase proteins and other systemic responses to inflammation.
227 N. Engl. J. Med.. 340, 448-454.
228 Gardner, I.A., Greiner, M. 2006. Receiver-operating characteristics curves and likelihood ratios:
229 iprovements over traditional methods for the evaluation and application of veterinary clinical
230 pathology tests. Vet. Clin. Pahol. 35, 8-17.
231 Kjelgaard-Hansen, M., Jensen, A.L., Kristensen, A.T., 2003. Evaluation of a commercially
232 available human C-reactive protein (CRP) turbidometric immunoassay for determination of
233 canine serum CRP concentration. Vet. Clin. Pathol. 32, 81-87.
234 Kuribayashi, T., Shimada, T., Matsumoto, M., Kawato, K., Honjyo, T., Fukuyama, M., Yamamoto,
235 Y., Yamamoto, S., 2003. Determination of serum C-reactive protein (CRP) in healthy beagle
236 dogs of various ages and pregnant beagle dogs. Exp. Anim. 52, 387-390.
237 Kutzler, M.A., Mohammed, H.O., Lamb, S.V., Meyers-Wallen, V.N., 2003. Accuracy of canine
238 parturition date prediction from the initial rise in preovulatory progesterone concentration.
239 Theriogenology. 60, 1187-1196.

240 Leong, A.S., Norman, J.E., Smith, R., 2008. Vascular and myometrial changes in the human uterus
241 at term. Reprod. Sci 15, 59-65.
242 Mendelson, C.R., Condon, J.C., 2005. New insights into the molecular endocrinology of parturition.
243 J. Steroid Biochem. Mol. Biol. 93, 113-119.
244 Onclin, K., Verstegen, J.P., 1997. Secretion patterns of plasma prolactin and progesterone in
245 pregnant compared with nonpregnant dioestrous beagle bitches. J Reprod.Fertil.Suppl. 51, 203-
246 208.
247 Rota, A., Charles, C., Starvaggi Cucuzza, A., Pregel, P., 2015. Diagnostic Efficacy of a single
248 progesterone determination to assess full-term pregnancy in the bitch. Reprod. Domest. Anim.
249 50, 1028-1031.
250 Skarżyńska, E., Zborowska, H., Jakimiuk, A.J., Karlińska, M., Lisowska-Myjak, B., 2018.
251 Variations in serum concentrations of C-reactive protein, ceruloplasmin, lactoferrin and
252 myeloperoxidase and their interactions during normal human pregnancy and postpartum period.
253 J. Trace Elem. Med. Biol. 46, 83-87.
254 Thomson, A.J., Telfer, J.F., Young, A., Campbell, S., Stewart, C.J., Cameron, I.T., Greer, I.A.,
255 Norman, J.E., 1999. Leukocytes infiltrate the myometrium during human parturition: further
256 evidence that labour is an inflammatory process. Hum. Reprod. 14, 229-236.
257 Ulutas, P.A., Musal, B., Kiral, F., Bildik, A., 2009. Acute phase protein levels in pregnancy and
258 oestrus cycle in bitches. Res. Vet. Sci. 86, 373–376.
259 Vannucchi, C.I., Mirandola, R.M., Oliveira, C.M., 2002. Acute-phase protein profile during
260 gestation and diestrous: proposal for an early pregnancy test in bitches. Anim. Reprod. Sci. 74,
261 87-99.
262 Wierzchosławski, K., Kwit, K., Pejsak, Z., Pomorska-Mólb, M., 2018. Selected serum acute-phase
263 proteins in peripartum sows and evaluation of their diagnostic usefulness. Anim. Reprod. Sci.
264 191, 44-55.

Yamashita, K., Fujinaga, T., Okumura, M., Takiguchi, M., Tsunoda, N., Mizuno, S., 1991. Serum C-reactive protein (CRP) in horses: the effect of aging, sex, delivery and inflammations on its concentration. J. Vet. Med. Sci. 53, 1019-1024.

TABLE LEGENDS

Table 1 Mean CRP concentration (mg/dl) from five days before to two days after parturition (day 0).

FIGURE LEGENDS

Fig. 1. Distribution of samples along the eight days of observation.

Fig. 2. Different mean CRP concentration (mg/dl) in pluriparous (n=14) and primiparous (n=14) bitches (*P*=0.044).

Fig. 3. Different mean CRP concentration (mg/dl) in bitches with litters of different size (*P*=0.0036). Different letters mean significant differences (*P*<0.01).

Number of bitches for each category of litter size: <4 puppies n=6; 4-8 puppies n=15; > 8 puppies n=7

Fig. 4. Mean progesterone concentration (ng/ml) from five days before to two days after parturition (day 0). Different letters mean significant different values (*P*<0.01).

Fig. 5. Association between CRP (mg/dl) and progesterone (ng/ml)

Fig. 6. Receiver Operating Characteristic (ROC) curve of CRP concentration to distinguish between bitches at term/not at term: the area under the curve (AUC) was 0.73 with a 95% CI (0.591-0.835; *P*=0.001).